REMARKS

I. Status of the Application

Claims 1-17 are pending in the application. New claim 18 has been added. Claims 1-10 stand rejected under 35 U.S.C. §112, first paragraph for failing to comply with the written description requirement. Claim 13 stands rejected under 35 U.S.C. §112 first paragraph for lacking enablement. Claims 1-17 stand rejected under 35 U.S.C. §112, second paragraph for being indefinite. Claims 1, 4, 5, 7, 8 and 10-14 stand rejected under 35 U.S.C. §102(b) as being anticipated by Walsh et al. (2000) *Bone* 27:185. Claims 1-5, 7, 8, 10, 11 and 14-17 stand rejected under 35 U.S.C. §102(b) as being anticipated by Scheven et al. (1995) *Journal of Bone and Mineral Research* 10:874. Claims 1-5 and 7-11 stand rejected under 35 U.S.C. §102(b) as being anticipated by Locklin et al. (1999) *Cell Biology International* 23:185. Claim 11 stands rejected under 35 U.S.C. §103(a) as being unpatentable over Walsh et al. in view of Nefussi et al. (1997) *Journal of Histochemistry and Cytochemistry* 45:493; and over Walsh et al. in view of Candeliere et al. (2001) *Bone* 28:351.

Applicant has amended the claims to more clearly define and distinctly characterize Applicant's novel invention. Support for the amendments can be found in the specification and the claims as originally filed. Specifically, support for the amendment to claim 17 to recite "observed in the presence of UV light" can be found in the specification at least at page 6, lines 6-8, where Applicants teach that the reaction product obtained after conversion of para-nitro phenyl phosphate by ALP may be detected using UV. Claims 1, 3 and 7-17 were amended to address formal matters. Support for new claim 18 can be found at least in claim 16 as originally filed.

The amendments presented herein add no new matter. Applicants respectfully request

entry and consideration of the foregoing remarks, which are intended to place this case in condition for allowance.

II. Formal Matters

At page 2, paragraph 4 of the instant Office Action, the Examiner has objected to the abstract of the disclosure "because it contains legal phraseology." In response, Applicants have amended the abstract to remove legal phraseology. Accordingly, Applicants request that this objection be withdrawn.

At page 2, paragraph 5 of the instant Office Action, the Examiner has objected to the title as not being descriptive. The Examiner helpfully suggests that the title "predicting the capacity of a cell population to induce bone formation" be used. In response, Applicants have amended the title to recite "predicting cell activity the capacity of a cell population to induce bone formation". Accordingly, Applicants request that this objection be withdrawn.

At page 2, paragraph 6 of the instant Office Action, the Examiner has noted the use of trademarks and requested that the trademark be capitalized and accompanied by the generic terminology. In response, Applicants have amended the specification to recite trademark symbols and to include generic terminology. Accordingly, Applicants request that this objection be withdrawn.

At page 3, paragraph 2 of the instant Office Action, the Examiner has objected to claim 16 for reciting "AS-B1 phosphate" because it is misspelled. In response, Applicants have amended the claim to recite "AS-BI," as helpfully suggested by the Examiner. Accordingly, Applicants request that this objection be withdrawn.

III. The Specification Provides Adequate Written Description for Claims 1-10

At page 3, paragraph 4 of the instant Office Action, claims 1-10 stand rejected under 35 U.S.C. §112, first paragraph for failing to comply with the written description requirement. The Examiner asserts that to provide adequate written description and evidence of possession of a claimed genus, the specification must provide sufficient distinguishing characteristics of the genus. The Examiner is of the opinion that there is no structural/functional basis provided by the prior art or the instant specification for one of skill in the art to envision the set of bone-specific proteins from any vertebrate species. The Examiner concludes that one of skill in the art would not have been able to envision a representative number of bone-specific proteins to describe the broad genus encompassed by the rejected claims, and that one of skill in the art would have reasonably concluded that Applicants were not in possession of the claimed invention for claims 1-10. Applicants respectfully traverse this rejection.

The first paragraph of 35 U.S.C. § 112 requires that the specification provide a written description of the claimed invention:

> [t]he specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The purpose of the written description requirement is to ensure that the specification conveys to those skilled in the art that the applicants possessed the claimed subject matter as of the filing date sought. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 U.S.P.Q.2d (BNA) 1111, 1117 (Fed. Cir. 1991). With respect to a claimed genus, the U.S. Patent and Trademark Office's Written Description Guidelines state:

> The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by . . . disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus . . . .

66 Fed. Reg. 1099, 1106 (January 5, 2001), internal reference omitted, approved in *Enzo Biochem, Inc. v. Gen-Probe Incorporated*, 296 F.3d 1316, 1325, 63 U.S.P.Q.2d (BNA) 1609, 1613 (Fed. Cir. 2002).

The specification describes a sufficient number of representative species having the recited characteristics to describe the recited genera. Applicants explicitly disclose the bone-specific proteins alkaline phosphatase, osteocalcin, bone sialoprotein, osteopontin and osteonectin (page 5, lines 3). Applicants respectfully submit that this disclosure corresponds to a representative number of bone-specific proteins known in the art at the time of filing, because at the time of filing (June 2001), the bone-specific proteins recited in the instant specification represented at least a majority of the bone-specific proteins known. Further, Applicants teach that bone-specific proteins can be expressed by bone marrow stromal cells, and that such expression may be stimulated by contacting the stromal cells with an osteogenic stimulation factor (page 5, lines 1-8). Finally, Applicants submit that "bone-specific protein" is an art-recognized term, and that one of skill in the art would immediately understand a bone-specific protein to be one that has the characteristic of being associated with bone (discussed further below).

Accordingly, Applicants submit that the claimed invention is described with sufficient particularity to demonstrate that Applicants had possession of the claimed invention, namely

bone-specific proteins. Therefore, Applicants respectfully request that the rejections of claims 1-10 under 35 U.S.C. § 112, first paragraph be reconsidered and withdrawn.

IV. Claim 13 is Enabled

At page 5, paragraph 3 of the instant Office Action, claim 13 stands rejected under 35 U.S.C. §112, first paragraph as lacking enablement. The Examiner states that hybridoma B4-78 is encompassed by the definitions for biological material set forth in 37 C.F.R. §1.801, and that it must be obtainable by a reproducible method or otherwise be known and readily available to the public as detailed in 37 C.F.R. §§1.801 through 1.809. The Examiner is of the opinion that it is unclear whether this material is known and readily available to the public and that availability of the hybridoma is deemed necessary to satisfy the enablement provisions of 35 U.S.C. §112. Applicants respectfully traverse this rejection.

Applicants respectfully submit that hybridoma B4-78 was publicly available in the art at the time of filing as evidenced at page 9, line 5 of Applicants' specification where Applicants teach that the hybridoma was "obtained from the Developmental Studies Hybridoma Bank (University of Iowa, USA)" and in Walsh et al. (see page 186, Antibodies section). Website materials from the Developmental Studies Hybridoma Bank at the University of Iowa pertaining to ordering this publicly available hybridoma are provided as attachment A. Accordingly, Applicants request that the rejection of claim 13 under 35 U.S.C. §112, first paragraph as lacking enablement be reconsidered and withdrawn.

V. Claims 1-17 Are Definite

At page 7, second paragraph, claims 1-17 stand rejected under 35 U.S.C. §112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Applicants respectfully traverse these rejections.

The second paragraph of 35 U.S.C. § 112 states that:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

It is well settled that a claim must "reasonably apprise those skilled in the art both of the utilization and scope of the invention." *Georgia-Pacific Corp. v. United States Plywood Corp.*, 258 F.2d 124, 134-38, 118 U.S.P.Q. 122, 130 (2d Cir. 1958), *cert. denied*, 358 U.S. 884 (1958). Claims 1-17 meet this standard.

The Office Action asserts that claims 1, 11 and 12 are vague and indefinite in that the metes and bounds of the term "bone-specific protein" are unclear. The Examiner states that the term is unclear in that it can be interpreted to mean a protein that is exclusively expressed in bone and is not expressed in any other tissue at any time in development, or it can be interpreted to mean a protein that is a marker for osteoblast differentiation. Applicants respectfully disagree.

Applicants respectfully submit that the term "bone-specific protein" is well known in the art to mean a protein that is associated with bone. Applicants teach that bone-specific proteins include proteins such as alkaline phosphatase, osteocalcin, bone sialoprotein, osteopontin and osteonectin (page 5, lines 2-5). The term "bone-specific protein" is commonly used in the art to refer to various bone-associated proteins such as osteonectin, osteocalcin, type I collagen and CMF-608. See, e.g., Termine et al. (1981) Cell 26:99; Johnson (2001) Clin. Geriatrics Vol. 9, Issue 1, page 4; Thomas et al. (2002) *Proc. Natl. Acad. Sci. USA* 99:1972, page 1976, right

column and page 1972, abbreviations; Segev (2004) *Bone* 34:246 (Attachments B-E). Accordingly, those skilled in the art would easily understand the term bone-specific protein as the term reasonably conveys to one skilled in the art what the invention is. Applicants respectfully request withdrawal of the rejection.

The Examiner is of the opinion that the term "based on α-MEM" in claim 7 is a relative term which renders the claim indefinite. Without acquiescing to the rejection, Applicants respectfully submit that claim 7 has been amended to recite "comprising α-MEM." Accordingly, Applicants respectfully request that the rejection of claim 7 under 35 U.S.C. §112, second paragraph be reconsidered and withdrawn.

The Examiner is of the opinion that claim 7 is vague and indefinite in that the metes and bounds of the phrase "wherein the medium further comprises L-ascorbic acid 2-phosphate, an antibiotic, serum, and/or a growth factor" are unclear. Applicants respectfully submit that claim 7 does not recite this language. Applicants will apply their response to claim 8, which does recite this language. Without acquiescing to the rejection, Applicants respectfully submit that claim 8 was amended to recite "one or more of the group consisting of L-ascorbic acid 2-phosphate, an antibiotic, serum, and a growth factor". Accordingly, Applicants respectfully request that this rejection be withdrawn.

The Examiner is of the opinion that claim 16 is indefinite for reciting the term "preferably" as it renders the claim indefinite. Without acquiescing to the rejection, Applicants respectfully submit that claim 16 has been amended to remove the language "preferably fast blue RR." Accordingly, Applicants request that this rejection be reconsidered and withdrawn.

The Examiner is of the opinion that claim 17 is indefinite for reciting the trademark/trade name Sigma 104® phosphate substrate. The Examiner states that the trademark/trade name is

used to identify/describe para-nitrophenyl phosphate (pNPP) disodium hexahydrate and, accordingly, the identification/description is indefinite. The Examiner further rejects claim 17 as vague and indefinite in that the metes and bounds of the phrase "and the reaction product is reacted further with Sigma 104R phosphatase substrate and subsequently detected by UV" is unclear. Without acquiescing to the Examiner's rejection Applicants respectfully submit that claim 17 has been amended to remove "reacted further with Sigma 104R phosphatase substrate." Accordingly, Applicants request that these rejections be withdrawn.

VI. Claims 1, 4, 5, 7, 8 and 10-14 Are Novel Over Walsh et al.

At page 9, paragraph 3 of the instant Office Action, claims 1, 4, 5, 7, 8, 10-13 and 14 stand rejected under 35 U.S.C. §102(b) as being anticipated by Walsh et al. (2000) *Bone* 27:185 as evidenced by Cheng et al. (1994) *Endocrinology*, 134:277. The Examiner asserts that Walsh et al. teaches a method of determining the osteogenic potential of a population of cultured cells. Applicants respectfully traverse this rejection. Applicants respectfully submit that for a reference to anticipate a claim, the reference must teach every element of the claim.

Claim 1 and claims depending therefrom are directed to a method of ***determining in vitro*** the ***capacity*** of a cell population ***to induce bone formation in vivo*** comprising the steps of providing a sample of a cell population, dividing said sample into a first and a second part, culturing the first part in the presence of an osteogenic stimulation factor, culturing the second part in the absence of an osteogenic stimulation factor, determining degrees of expression of a bone-specific protein, and ***comparing the degrees of expression of the bone-specific protein of the first part and the second part thereby providing a measure for the capacity of the bone cell population to induce bone formation in vivo.***

Applicants' claimed method enables one of skill in the art to predict, using an *in vitro* assay, the ability of a cell population to induce bone formation *in vivo*. Applicants' claimed method is based in part on Applicants' discovery that the capacity of a cell population to form bone *in vivo* ***does not always correlate*** with *in vitro* expression of markers such as procollagen I (PCI), osteopontin (OP), or alkaline phosphatase (ALP) by the cell population. Indeed, Applicants established that the capacity of cells to express PCI, OP or ALP in culture did not correlate with osteogenesis in immunodeficient mice. *See* specification page 14, lines 5-22 and Table 2. Surprisingly, however, Applicants have discovered that the ability of a cell population to induce bone formation *in vivo* can be determined by observing *in vitro* expression of a bone-specific protein by cells that have been exposed to an osteogenic stimulation factor. Applicants have determined that the ***degree of in vitro expression*** of a bone-specific protein by cells in response to exposure to an osteogenic factor ***correlates*** with the ***ability*** of the cell population ***to induce bone formation in vivo***.

Applicants' claimed method provides an accurate, facile technique whereby one of skill in the art may ascertain the efficacy of a cell population for inducing bone formation *in vivo*. Applicants' claimed method provides for the detection of cultures with low osteoconductivity prior to their implantation into a recipient, and thus can reduce the frequency in which bone induction *in vivo* is not achieved. Applicants' claimed invention provides a distinct advantage over methods known in the art at the time of filing in which the ability of the cells to promote bone formation could only be established *in vivo* after a lengthy and cumbersome procedure.

Walsh et al. is directed to expanding cells having enhanced osteogenic potential by culturing bone marrow mononuclear cells in the presence of fibroblast growth factor-2 (FGF-2) (abstract, page 186, first full paragraph). Walsh et al. teaches that treatment of bone marrow

mononuclear cells with FGF-2 "markedly increased their proliferative potential and that of their immediate progeny" (abstract). Walsh et al. is directed to a method of ***enriching*** a population of cells to preferentially produce a group of cells expressing certain developmental markers such as STRO-1 and alkaline phosphatase (page 186, first full paragraph). Walsh et al. fails to assess ***in vivo*** the ability of their enriched cells to induce bone formation. Nor does Walsh et al. correlate the ***degree*** to which cells ***respond to an osteogenic factor*** by ***expressing a bone-specific protein*** with the ***ability*** to induce bone formation *in vivo*. Walsh et al. merely assays the induction of marker (i.e., STRO-1 AND AP) expression, which Applicants have discovered does not always correlate with the ability to induce bone formation *in vivo*. Applicants' claimed invention is directed to a method of determining the *in vivo* osteogenic potential of a population of cells based upon their response to exposure to an osteogenic stimulation factor, not expanding the cells to selectively increase a population of cells having osteogenic potential, as taught by Walsh et al.

Thus, Walsh et al. fails to teach or suggest each and every element of the claimed invention. Accordingly, Applicants request that the rejection of claims 1, 4, 5, 7, 8, 10-13 and 14 under 35 U.S.C. §102(b) as being anticipated by Walsh et al. be reconsidered and withdrawn.

VII. Claims 1-5, 7, 8, 10, 11 and 14-17 Are Novel Over Scheven et al.

At page 11, paragraph 1 of the instant Office Action, claims 1-5, 7, 8, 10, 11 and 14-17 stand rejected under 35 U.S.C. §102(b) as being anticipated by Scheven et al. (1995) Journal of Bone and Mineral Research 10:874. The Examiner asserts that Scheven et al. teaches the establishment of human osteoblast cultures from trabecular bone explants of femoral heads obtained from orthopedic surgery. Applicants respectfully traverse this rejection.

Scheven et al. is directed to studying the effects of methotrexate (MTX) on osteoblasts *in vitro* in the presence of vitamin D3 in order to determine the effects of MTX on bone metabolism and remodeling (abstract). Scheven et al. teaches that vitamin D3 causes suppression of osteoblast proliferation and a strong induction of osteoblastic differentiation as determined by AP and osteocalcin expression in cultured cells (page 876, second full paragraph). Scheven et al. fails to assess ***in vivo*** the ability of their cultured cells to induce bone formation. Nor does Scheven et al. correlate the ***degree*** to which cells respond to an osteogenic factor by expressing a bone-specific protein with the ***ability*** to induce bone formation *in vivo*, as required by the instant claims. In fact, Scheven et al. states that the "physiological and clinical relevance of our in vitro findings is not yet clear" (page 879, right column, first paragraph).

Thus, Scheven et al. fails to teach or suggest each and every element of the claimed invention. Accordingly, Applicants request that the rejection of claims 1-5, 7, 8, 10, 11 and 14-17 under 35 U.S.C. §102(b) as being anticipated by Scheven et al. be reconsidered and withdrawn.

VIII. Claims 1-5 and 7-11 Are Novel Over Locklin et al.

At page 11, paragraph 3 of the instant Office Action, claims 1-5 and 7-11 stand rejected under 35 U.S.C. §102(b) as being anticipated by Locklin et al. (1999) *Cell Biology International* 23:185. The Examiner asserts that Locklin et al. teaches the isolation of primary cultures of bone marrow cells obtained from patients undergoing total hip replacement surgery and the growth of the cultures in alpha-minimum essential medium containing fetal calf serum, penicillin and streptomycin. Applicants respectfully traverse this rejection.

Locklin et al. is directed to investigating the effects of TGFβ, bFGF and dexamethasone on cultured human bone marrow fibroblastic cells (abstract). Locklin et al. assays alkaline phosphatase activity and matrix production in cultured human bone marrow fibroblasts exposed to TGFβ and bFGF (pages 188 and 189). Locklin et al. fails to assess ***in vivo*** the ability of their cultured cells to induce bone formation. Nor does Locklin et al. correlate the ***degree*** to which cells respond to an osteogenic factor by expressing a bone-specific protein with the ***ability*** to induce bone formation *in vivo*, as required by the instant claims.

Thus, Locklin et al. fails to teach or suggest each and every element of the claimed invention. Accordingly, Applicants request that the rejection of claims 1-5 and 7-11 under 35 U.S.C. §102(b) as being anticipated by Locklin et al. be reconsidered and withdrawn.

IX. Claim 11 is Nonobvious Over Walsh et al. In View of Nefussi et al. and Walsh et al. In View of Candeliere et al.

At page 13, paragraph 1 of the instant Office Action, claim 11 stands rejected under 35 U.S.C. §103(a) as being obvious over Walsh et al. in view of Nefussi et al. (1997) *The Journal of Histochemistry & Cytochemistry* 45:493. The Examiner admits that Walsh et al. does not teach the quantification of bone sialoprotein or osteonectin expression. The Examiner is of the opinion that it would have been obvious to modify the teachings of Walsh et al. to include the antibodies and detection methods taught by Nefussi et al. because Walsh et al. teach it is within the skill of the art to use antibodies to detect markers of osteogenic differentiation and because Nefussi et al. teach the use of antibodies to detect the mineralized matrix formed by the cultured cells.

At page 14, paragraph 2 of the instant Office Action claim 11 stands rejected under 35 U.S.C. §103(a) as being obvious over Walsh et al. in view of Candeliere et al. (2001) Bone 28:351. The Examiner admits that Walsh et al. does not teach the quantification of bone

sialoprotein, osteocalcin and osteopontin. The Examiner is of the opinion that it would have been obvious to modify the teachings of Walsh et al. to include the antibodies taught by Candeliere et al. because Walsh et al. teach it is within the skill of the art to use antibodies to detect markers of osteogenic differentiation and because Candeliere et al. teaches the use of antibodies to further characterize the cells of the osteoblast lineage. Applicants respectfully traverse these rejections.

Walsh et al. fails to teach or suggest the claimed invention for at least the reasons set forth above. The secondary references fail to cure the deficiencies of Walsh et al.

Nefussi et al. is directed to the *in vitro* characterization of rat calvaria bone cells over a 15 day culture period (summary; page 494, materials and methods, first paragraph). Nefussi et al. fails to assess ***in vivo*** the ability of their cultured cells to induce bone formation. Nor does Nefussi et al. correlate the ***degree*** to which cells respond to an osteogenic factor by expressing a bone-specific protein with the ***ability*** to induce bone formation *in vivo*, as required by the instant claims. Accordingly, the combination of Walsh et al. and Nefussi et al. fails to render the claimed invention obvious.

Candeliere et al. is directed to the study of mRNA and protein expression levels in different developmental or maturational zones in calvaria cells from twenty one day old fetal rats (page 352, second full paragraph). Candeliere et al. teaches *in situ* hybridization and immunohistochemistry of coronal and cryostat sections obtained from fetal rats (page 352, materials and methods). Candeliere et al. fails to assess ***in vivo*** the ability of ***cultured cells*** to induce bone formation. Nor does Candeliere et al. correlate the ***degree*** to which cells respond to an osteogenic factor by expressing a bone-specific protein with the ***ability*** to induce bone

formation *in vivo*, as required by the instant claims. Accordingly, the combination of Candeliere et al. and Nefussi et al. fails to render the claimed invention obvious.

Thus, the art cited by the Examiner, alone or in combination, fails to teach each and every element of the claimed invention. Accordingly, Applicants respectfully request that the Examiner withdraw the rejections of claim 11 under 35 U.S.C. §103(a).

X. Conclusion

Having addressed all outstanding issues, Applicant respectfully requests reconsideration and allowance of the case. To the extent the Examiner believes that it would facilitate allowance of the case, the Examiner is requested to telephone the undersigned at the number below.

Respectfully submitted,

Dated: March 22, 2005

Judith Stone-Hulslander, Reg. No. 55,652
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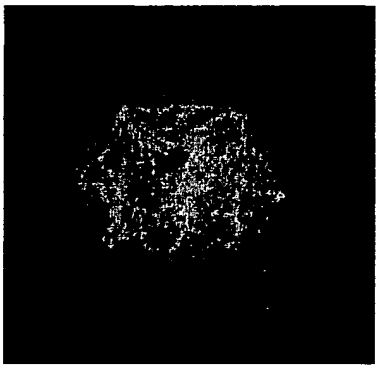

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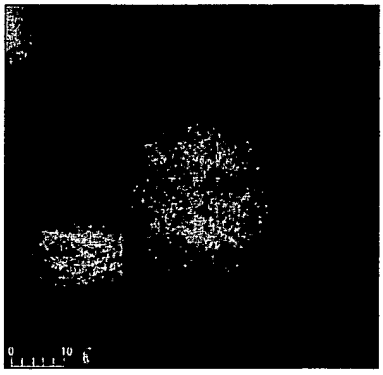

Microtubules

INVESTIGATOR

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IMMUNOGEN

Substance	human bone alkaline phosphatase
Name	ALP, EC 3.1.3.1
Origin	human bone
Chemical Composition	
Developmental Stage	

IMMUNIZATION PROTOCOL

Donor Animal	
Species	mouse
Strain	BALB/c
Sex	female
Organ and tissue	spleen
Immunization	
Dates immunized	
Amount of antigen	100 μg
Route of immunization	IP
Adjuvant	Freund's

FUSION

Date	
Myeloma cell line	
Species	mouse
Designation	NS1

MONOCLONAL ANTIBODY

Isotype	IgG1, kappa light chain
Specificity	human bone and liver ALP
Cell binding	
Immunohistology	
Antibody competition	
Species Specificity	human, mouse

ANTIGEN

Chemical properties	
Molecular weight	
Characterization	
Immunoprecipitation	
Immunoblotting	yes
Purification	
Amino acid sequence analysis	
Functional effects	
Immunohistochemistry	

PUBLICATIONS :

Lawson, G.M., Katzman, J.A., Kimlinger, T.K., and O'Brien, J.F. (1985). Isolation and preliminary characterization of a monoclonal antibody that interacts preferentially with the liver isoenzyme of human alkaline phosphatase. Clin. Chem. 31, 381-385.

Dorheim, M.-A., Sullivan, M., Dandapani, V., Wu, X., Hudson, J., Segarini, P.R., Rosen, D.M., Aulthouse, A.L., and Gimble, J.M. (1993). Osteoblastic gene expression during adipogenesis in hematopoietic supporting murine bone marrow stromal cells. J. Cell. Phys. 154, 317-328.

About the DSHB

Under the auspices of the National Institute of Child Health & Human Development, the Developmental Studies Hybridoma Bank was established in 1986 to supply investigators with monoclonal antibodies useful for studies in developmental and cell biology. They may be ordered as tissue culture supernatants, ascites, or concentrate; selected hybridomas are also available as frozen or growing cells. The DSHB is administered by David R. Soll, Ph.D. at The University of Iowa.

Contacting the DSHB

For information about the DSHB or information on contributing hybridomas to the DSHB:

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Queries about Orders, Product Availability and Technical Information

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Citing the DSHB - Acknowledgments Statement

We have been asked by NICHD to ensure that all investigators include an acknowledgment in publications that benefit from the use of the DSHB's products. We suggest that the following statement be used:

"The (hybridoma or monoclonal antibody) developed by [Investigator (s)] was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242."

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Note: Performing your original search, ***"bone specific protein"***, in PubMed will retrieve ***49*** citations.

1: Cell. 1981 Oct;26(1 Pt 1):99-105.

Osteonectin, a bone-specific protein linking mineral to collagen.

Termine JD, Kleinman HK, Whitson SW, Conn KM, McGarvey ML, Martin GR.

Osteonectin is a 32,000 dalton bone-specific protein that binds selectively to both hydroxyapatite and collagen. When osteonectin is bound to insolubilized type I collagen, the resultant complex binds synthetic apatite crystals and free calcium ions. The osteonectin-collagen complexes also nucleate mineral phase deposition from metastable balanced salt solutions, Antibodies to osteonectin cross-react with bone and, to a lesser extent, dentin, but not with other tissues. The protein is localized to mineralized bone trabeculae and occurs at higher levels in the matrix than in the cells of bone. These studies suggest that osteonectin is a tissue-specific protein, linking the bone mineral and collagen phases, perhaps initiating active mineralization in normal skeletal tissue.

PMID: 7034958 [PubMed - indexed for MEDLINE]

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Malnutrition

By Larry E. Johnson, M.D., Ph.D.

Undernutrition and decreased nutrient reserves are estimated to affect substantial numbers of elderly persons. Although not an inevitable side effect of aging, undernutrition can result from a variety of common and often interacting factors. The presence of any of these should be a warning that malnutrition may be present. Biochemical causes of decreased intake in older persons include a decrease in the opioid food intake drive, an increase in cholecystokinin, and a decrease in nitric oxide, which is involved in relaxation of the fundus of the stomach. Separating the effects of normal aging from those of intercurrent disease remains as difficult here as in many areas of geriatrics. Malnutrition may increase the risk for many problems commonly found in elderly persons, for example, decreased resistance to infection, poor wound healing and increased skin friability, osteopenia, anemia, certain cardiovascular diseases, cataracts, and age-associated macular degeneration. In older adults, undernutrition often precedes hospitalization.

Gastrointestinal functions change minimally with normal aging. Gastric acid secretion is diminished in 10 to 15% of older adults. Loss of olfactory function and decline in taste threshold sensitivity may adversely affect the older adult's nutritional status.

Macronutrients

Calories Energy intake progressively decreases with age. The energy intake of men both in the Baltimore Longitudinal Study of Aging and the National Health and Nutrition Examination Study (NHANES) fell from 2,700 kcal at ages 23 to 34 to 1,800 to 2,100 kcal at ages 65 to 80. One-third of this decrease was due to a lower metabolic rate. Metabolically active skeletal muscle declined from 45% of body weight in young adults to about 27% by age 70 years; two thirds of the reduced energy requirement was due to decreased physical activity. Of persons 65 years of age and older, 16% eat less than 1,000 kcal a day.

Protein Daily protein requirements are difficult to establish. Young and middle-aged adults require approximately 0.8 g/kg. Protein requirements may increase slightly with age, to 1.0 to 1.25 g/kg in healthy elderly persons. Low-calorie diets require more protein than high-calorie diets to achieve nitrogen balance. The protein intake of undernourished patients and of those in catabolic states following surgery, infections, or trauma should be increased. The Baltimore Longitudinal Study of Aging did not show a relationship between high protein intake and impairment of creatinine clearance. Also, although high protein intakes can lead to increased

urinary calcium excretion, there is no evidence that this is a factor contributing to osteoporosis in the United States. Physical exercise may improve nitrogen balance.

Fat Fat calories should not exceed 30% of total daily calorie intake. It is not known if there are changes in fat requirements with aging. However, intriguing information about health aspects of different kinds of fat is emerging. Total fat intake is not closely associated with coronary artery disease risk. Trans fatty acids (called hydrogenated or partially hydrogenated fats on food labels) increase low-density lipoprotein and decrease high-density lipoprotein levels more than saturated fats do. Monounsaturated fats (like olive oil) and polyunsaturated fats high in omega-3 fatty acids (such as canola, soybean, flaxseed, and fish oils) are associated with decreased fatal coronary artery disease risk. Advising older patients to replace saturated fats and trans fatty acids with mono- and polyunsaturated fats may result in healthier outcomes and better dietary adherence than merely recommending a low-fat diet.

Water Dehydration is the most common fluid and electrolyte disturbance in older persons. Many diseases affecting the mental or physical capabilities of older adults reduce their access to fluids or their ability to recognize or express thirst. In addition, a decrease in thirst perception and reduced response to serum osmolality (hypodipsia), as well as decreased ability to concentrate urine following fluid deprivation, occurs in normal aging. It may be necessary to encourage fluids for bedbound or institutionalized elderly persons; giving precise volume guidelines to care givers or nurses may be more useful than merely asking them to "push fluids." A recommended daily intake of 2 L is equivalent to about 80 mL/hr.

Sodium The minimum intake of sodium for all adults, including elderly persons, is 500 mg/day (or about 1.3 g sodium chloride). Most persons in the U.S. are accustomed to much higher salt intakes and may find a low-salt diet unpalatable, which is why undernutrition or nonadherence may occur when sodium intake is restricted. It is important to confirm whether sodium restriction is absolutely necessary. Obese hypertensive persons may be more likely to respond to salt restriction than those of average weight. Postural hypotension may result from extreme salt restriction.

Fiber A meta-analysis of 13 case-control studies, correcting for ascorbic acid and carotenoid intake, concluded that colorectal cancer risk decreases as fiber ingestion increases. However, increased fiber intake does not decrease recurrence of polyps over the short term. Higher intakes of insoluble cereal fiber are associated with decreased heart attack risk in men. The typical American diet contains 8 to 17 g of total fiber; increasing fiber intakes (gradually, to avoid abdominal discomfort) to 20 to 35 g is currently recommended. Older adults can be encouraged to improve their fiber intake by advising them to eat fruits and vegetables that "crunch." Increasing dietary fruits and vegetables lowers blood pressure. The phytate associated with some natural fiber sources (cereals, legumes, vegetables) can impair calcium, zinc, and iron absorption, but usually is not clinically significant.

Micronutrients

Vitamins The recommended dietary allowances (RDAs) published in 1989 have generated criticism. For example, the RDAs group all adults 51 and older in a single category. In addition, although RDAs have been influential in reducing the prevalence of severe deficiency states such as pellagra and beriberi, the RDAs for certain vitamins may be inadequate for optimum health. Decreased vitamin and mineral intake is most common in frail elderly persons but occurs even in relatively robust community-dwelling older adults. Many older adults consume little vitamin B6, B12, D, and folate, as well as calcium, magnesium and zinc. Severe vitamin-deficiency states are sufficiently rare that even when they present with classic manifestations, diagnosis may be delayed. More subtle deficiencies mimic or are masked by common age-associated comorbidity. Weakness, bone pain, skin disorders, cheilosis and stomatitis, increased susceptibility to infections, weight loss, and failure to thrive are manifestations of both undernutrition and many common disease states.

Vitamin C and the B vitamins (including folic acid) can become deficient over weeks or months, but fat-soluble vitamins A, D, and E, as well as those like vitamin B12 with efficient enterohepatic circulation and stores, require longer periods of deprivation to become deficient. Most B vitamins become deficient as a group. However, isolated B-vitamin deficiencies may occur, such as thiamin deficiency in alcoholics; pyridoxine deficiency due to specific antagonists such as isoniazid, carbidopa, and hydralazine; and folate deficiency caused by chronic trimethoprim, alcohol, or anticonvulsant use. A daily general multivitamin, without supplemental iron, can be recommended for older adults with virtually no side effects.

Vitamin B12 (cobalamin) Estimates of the prevalence of cobalamin deficiency in elderly persons range from 3 to 44%. Deficiency in elderly persons may be due to atrophic gastritis, Helicobacter pylori infection, or gastric and ileal surgery. Prolonged use of gastric antacids, H2-receptor antagonists, or proton pump inhibitors further decrease absorption and thus exacerbate the deficiency.

The serum vitamin B12 assay is currently the most practical and widely available measure for detecting cobalamin deficiency. Serum holotranscobalamin II may prove a better assay in the future. Macrocytosis and megaloblastic anemia are late and sometimes absent manifestations of deficiency. Slowly progressive dementias are only rarely related to vitamin B12 status or affected by vitamin replacement. Methylmalonic acid elevations can be detected in the urine and serum before the appearance of low serum cobalamin levels and can help detect tissue deficiency when the serum vitamin B12 level is still borderline low-normal (200 to 350 pg/mL). Oral vitamin B12 given in large doses (1,000 to 2,000 µg/day) is effective if the patient is otherwise asymptomatic. Serum vitamin B12 levels should be assessed more frequently with oral replacement to assure that absorption remains adequate.

Folate Folate deficiency is commonly linked to general malnutrition and alcohol abuse. It may also occur secondary to gut mucosal edema in chronic heart failure, or result from certain drug-nutrient interactions. As a diagnostic test when diet has not changed dramatically, serum folate is generally sufficient, although tissue stores are better estimated by red blood cell folate levels. Macrocytosis and anemia, as in vitamin B12 deficiency, appear late. Folate supplementation in the presence of cobalamin deficiency may mask the hematologic signs of vitamin B12 deficiency without reversing the continuing neurological deterioration. High blood homocysteine levels (> 10 to 15 µmol/L) are associated with a two- to 40-fold increased risk of coronary artery disease, cerebrovascular disease and stroke, and peripheral vascular disease. Much of the variance in homocysteine levels is due to folate intake; vitamin B12 and vitamin B6 intake appear to play lesser roles. Homocysteine concentrations have been found to be higher in persons with vascular dementia. Also, certain tests of cognitive function have been found to correlate with homocysteine concentrations. The role of folate supplementation to prevent or treat vascular disease, and the inverse association between coffee intake and homocysteine, are currently under investigation.

Vitamin D Poor vitamin D intake is common in older adults as sunlight exposure and consumption of fortified dairy products are often inadequate. Poor intake of dairy products also contributes to decreased calcium intake. This combination of calcium and vitamin D deficiencies leads to poor bone health and increased fracture risk. Adults should try to consume 1,500 mg of calcium and 400 to 800 IU of vitamin D daily. This may be difficult to do without supplementation, as one cup of fortified milk only has about 300 mg of calcium and 100 IU of vitamin D. Calcium carbonate is the least expensive supplement but is best absorbed when consumed with food. High fiber diets can interfere with its absorption. Other formulations, like calcium citrate, are well absorbed even on an empty stomach.

Vitamin D binding receptors are found in the prostate gland. A dose-response effect has been noted, as 1,25(OH)2D3 (calcitriol) decreases proliferation rates in prostate cancer cell lines, and calcitriol has been shown to slow prostate-specific antigen doubling time in early recurrent prostate cancer. Clinical studies are under way to further evaluate the interaction of vitamin D (and safer analogues) with prostate cancer, as well as other malignancies such as breast cancer and leukemia.

Vitamin K Vitamin K plays an incompletely understood role in bone metabolism. Vitamin K depletion that has no effect on blood coagulation can decrease the carboxylation of the bone protein osteocalcin (OC), producing undercarboxylated osteocalcin (ucOC). OC, whose function is currently unknown, is a bone-specific protein synthesized by osteoblasts. Its synthesis is affected by both vitamin D and vitamin K. High levels of ucOC correlate with osteopenia.

The RDAs for vitamin K are based on its role in blood coagulation, rather than on its role in bone metabolism. In dosages above the RDA (1 mg/day), vitamin K can decrease serum ucOC,

increase calcium absorption, and reduce urinary calcium loss in postmenopausal women. Administration of 45 mg of vitamin K daily to postmenopausal women with osteoporosis for one year has been found to increase cortical bone mass, although no improvement in lumbar bone mineral density was found. Whether vitamin K should be supplemented in persons at risk for osteopenia has not yet been determined.

Long-term use of vitamin K antagonists, such as oral anticoagulants and certain antibiotics, could theoretically reduce bone mineral content, although one study found that the bone density of chronic warfarin users did not differ from that of matched control persons.

Vitamins as Antioxidants The vitamins with substantial antioxidant properties are vitamins C and E, and the carotenoids, precursors to vitamin A. Riboflavin and selenium, as well as certain foods (e.g., garlic, soybeans, and green tea), also have antioxidant properties. Pro-oxidants, such as heme iron, and oxidative by-products are suspected of playing a role in many diseases, and perhaps aging itself, and antioxidants may be protective. Data on the beneficial effects of antioxidants are conflicting, and thus this subject remains controversial. Primarily epidemiologic studies suggest a protective role for antioxidant vitamin intakes, when ingested in quantities substantially above the current RDAs, in preventing age-related cataracts and age-related macular degeneration, and in reducing cardiovascular disease and mortality. Vitamin E may also play a role in slowing the progression of Alzheimer's disease. On the other hand, high intakes of vitamin C can enhance iron absorption and its release from tissue stores, increasing low-density lipoprotein oxidation and, perhaps, coronary artery disease risk. The role of antioxidants in carcinogenesis is also unclear. Several recent studies have found an increased incidence of lung cancer in middle-aged adults consuming beta-carotene supplements. The antioxidant vitamins likely have additional protective effects unrelated to their antioxidant properties. Foods that contain antioxidant vitamins often contain fiber and a variety of other phytochemicals that are probably beneficial (e.g., lycopenes in tomato paste may reduce the risk for prostate cancer).

Vitamin Supplementation Most persons, elderly or not, do not consume fruits and vegetables in sufficient quantities; a minimum intake of five servings daily has been recommended. Anyone who needs assistance with eating has a greatly increased risk of both macro- and micronutrient deficiency. The RDAs for most vitamins can be obtained from a diet rich in fruits and vegetables. Vitamin D can come from adequate sunshine exposure or fortified foods; vitamin B12 is primarily in meats; vitamin E is found naturally in vegetable oils. It is estimated that 30 to 60% of adults already take vitamin supplements with remarkably little toxicity. Patients should realize that supplementation is not a substitute for a balanced diet and that supplement use does not fully compensate for adverse risk behaviors. A caloric intake of less than 1,500 kcal/day is often vitamin deficient. The risks and benefits of supplements consumed in high concentrations remain unknown. They can be expensive.

Zinc Many studies of elderly persons suggest poor zinc intake is common. Zinc intake correlates with protein intake, which often is reduced in the frail elderly person; diuretics, diabetes mellitus, and inflammation increase urinary zinc loss, and various cytokines can alter zinc metabolism. Absorption of zinc decreases with aging. Zinc status is difficult to determine, as serum zinc levels correlate poorly with tissue levels. Zinc deficiency has been associated with impaired wound healing, hypogonadism, diarrhea, and decreased vision, olfaction, insulin, and immune function. Although severe zinc deficiency can result in hypogeusia, a double-blind study of patients with mild to moderate zinc deficiency did not show any improvement in hypogeusia with zinc supplementation. Early data suggest that age-related macular degeneration may be slowed by zinc supplementation. Persons taking prescription levels of zinc supplements (220 mg/day) may develop gastrointestinal upset or a sideroblastic anemia from impaired copper absorption. They should be closely monitored with a complete blood cell count every six months. High intakes of zinc may also interfere with the absorption of other vitamins and minerals and adversely affect cellular immunity. As with use of other "mega dose" micronutrients, new toxicities will continue to be recognized.

Nutritional Assessment Assessment of undernutrition and failure to thrive requires a careful history with a thorough medication review and social history, physical examination, and laboratory evaluation. Ideal body weight remains unknown for older adults. No single indicator of nutritional health, and especially no single laboratory test, is adequately sensitive or specific. The use of risk categories and various screening tools can help identify persons likely to benefit from further evaluation. For example, smoking and high alcohol intake are frequently correlated with poor nutrition. Involuntary weight loss exceeding 5% in one month, 7% in three months, and 10% in six months must trigger a complete evaluation.

The laboratory assessment of malnutrition generally includes a complete blood cell count (to look for nutritionally related anemias), albumin, and cholesterol determinations. All laboratory tests are affected by nonnutritional factors, and none alone is particularly sensitive or specific. Albumin levels change little with healthy aging. Albumin levels below 3.5 g/dL are associated with increased mortality in frail, institutionalized elderly. A cohort study of community-dwelling elderly persons (71 years and older) found a graded increase in all-cause mortality risk with albumin levels lower than 5.0 g/dL. Hypoalbuminemia in the presence of physical disability also identifies increasing mortality risk. Albumin synthesis is very sensitive to inflammatory conditions, and cytokines can suppress albumin synthesis in the liver. Thus, refeeding under these conditions may not be accompanied by an increased serum albumin. A cholesterol level under 160 mg/dL in the frail elderly person has also been shown to be a risk marker for increased mortality. Total lymphocyte count and anergy skin testing have not proven to be useful nutritional markers in older persons. Other serum protein markers have theoretical utility but are generally less clinically relevant. Transferrin, with a half-life of nine days, is influenced by iron status and antibiotics. Prealbumin (also called transthyretin or thyroxine-binding prealbumýn) has a half-life of about two days and may be useful in monitoring nutritional

interventions and refeeding. Severe stress or a nephrotic syndrome decreases the prealbumin level; corticosteroids may elevate it. Retinol-binding protein, fibronectin, insulin-like growth factor 1 (somatomedin C), C-reactive protein, and tumor necrosis factor remain experimental.

Persistent Undernutrition A 5% weight gain in previously malnourished institutionalized elderly persons is associated with decreased morbidity and mortality, whereas a 5% involuntary weight loss over one month is associated with almost five times greater one-year mortality. Strategies to increase nutritional intake are required in persons with persistent undernutrition. The role of depression as a cause of weight loss should be emphasized. Antidepressants associated with anorexia, particularly the selective serotonin reuptake inhibitors, may be effective treatment but require careful monitoring. The assistance of a nutritionist with geriatrics training can be valuable in assessing intake, modifying diet, and teaching and encouraging the patient and family. Multiple smaller feedings throughout the day may be effective. Patients who are losing weight may, surprisingly, respond when adequate hand feeding is instituted. Feeding undernourished patients anything they will eat, including "junk" foods, may help them regain their appetite for more healthy foods. Protein supplements may be associated with weight gain in some nursing-home residents who have lost weight. However, inadequate protein or vitamin intake may still occur. Many commercial supplements are now available; some are designed specifically for patients with certain medical problems, such as diabetes mellitus, renal failure, or pulmonary disease. If a patient tolerates cow's milk, instant breakfast powders mixed in milk are especially useful. Even persons who consider themselves lactose intolerant usually can drink up to two cups of milk daily without appreciable symptoms. When calculating fluid intake, one should remember that approximately 70% of the volume of these supplements is water.

Appetite stimulants are now being tested in frail elderly persons. Megestrol acetate, dronabinol, anabolic androgenic steroids, and psychostimulants have all been used in patients with cancer or acquired immunodeficiency syndrome (AIDS) to promote appetite and weight gain with limited success in increasing lean body mass or improving survival. Human growth hormone, although extremely expensive, has been found to be promising in treating undernutrition. Its value for the healthy older adult is currently under study and remains unknown. The targeting of specific cytokines involved in anorexia and cachexia is also being investigated.

Obesity The prevalence of obesity in the U.S. is substantial, although it progressively decreases after the age of 60. Longitudinal and cross-sectional surveys have shown that, in both black and white American men, body mass index (BMI) increases with age up to about 50 years, plateaus until about age 60, and then declines; this pattern seems to be delayed about a decade in women. BMI, usually given in units of kg/m2, is a standard measure of weight adjusted for height. Approximately 42% of both men and women between the ages of 60 and 69, and 37% between the ages of 70 and 79, are overweight (BMI > 25). For persons older than 80 years, 18% of men and 26% of women are overweight. Approximately 15% of older persons entering nursing homes are overweight. The prevalence of obesity has been increasing over

the past 30 years.

Aging in the U.S. is commonly associated with a decrease in lean body mass and a progressive increase in fat, with an age-related intra-abdominal redistribution of adipose mass even in healthy elderly persons and those with no change in weight. Women on hormone replacement therapy have less postmenopausal weight gain than do women not on therapy. Obesity in elderly persons is associated with coronary artery disease, hypertension, and diabetes mellitus, as well as degenerative joint disease, hepatic steatosis, gallbladder disease, gout, proteinuria, pulmonary function impairment, some cancers, and possibly immune dysfunction. Both high and low BMI increase the risk for functional impairment in elderly persons. Elevated BMI strongly predicts risk for symptomatic knee osteoarthritis in elderly women, and weight loss significantly lowers the osteoarthritis rate in women whose BMI exceeds 25.

All adipose tissue is not the same; that located around the waist and within the abdomen is more metabolically active (associated with increased free fatty acids in the portal system and potentially harmful lipid and insulin changes) than that in the thighs and buttocks. The waist-to-hip ratio (WHR), or waist circumference alone (men > 102 cm [> 40 inches]; women > 88 cm [> 35 inches]), surrogate measures of abdominal obesity, appear to be better predictors than BMI or percentage of body fat for coronary artery disease, hypertension, and mortality risk, at least in white Americans. Much remains to be learned about racial differences in fat patterning and their health consequences. Studies are conflicting regarding whether the BMI is predictive of mortality in black men, although the WHR appears to be. In contrast, neither BMI nor fat location ratio seems to predict mortality in black women except with extreme obesity. A recent study using computed tomography found that obese black women have 23% less visceral adipose tissue than white women with similar WHR, and this difference did not change with weight loss.

The Metropolitan Life Insurance Company weight-for-height reference tables (published in 1959, revised in 1983, but reflecting mortality in policyholders a decade earlier, and not age adjusted) and the Masters' tables (published in 1960 and age-adjusted to age 75) are now outdated and may have little applicability to current or future older persons and to minority populations. These data, of course, do not necessarily represent healthy weight goals. Normative data regarding body weights for most ethnic elderly populations in the U.S. are not available.

The body weight associated with greatest longevity remains unclear. When insurance data are analyzed, the BMI associated with least mortality seems to increase slowly from age 20 to age 69 (by 5.2 kg/m2 in men and 7.8 kg/m2 in women), and persons with lower or higher weights have increased mortality risk (the U-shaped curve). A competing hypothesis is that body weight and mortality are directly and linearly related (at least in white American men), that minimum mortality occurs at weights 10 to 20% below the U.S. average, and that what appears to be

excess mortality in the lean body habitus is bias from cigarette smoking and antecedent disease. A BMI of 27 or greater in late middle age increases the risk of coronary heart disease in late life by 70%. However, increased weight may be protective in certain instances; black women with a BMI < 22.6 have been found to have a much greater risk of hip fracture than heavier black women. In addition, overweight nursing home residents have lower mortality and fewer hip fractures and hospitalizations than residents of average weight. Mortality in underweight male residents is very high.

It is not known how aggressively one should encourage weight loss in obese elderly persons. Those with hypertension, diabetes mellitus, arthritis, and perhaps hyperlipidemia, but who are otherwise healthy, are most likely to benefit from nutritional counseling and modest weight reduction. Weight-reducing programs require a combination of diet modification and creative exercise, as well as close follow-up to prevent undernutrition. Involuntary weight loss in any older adult, including the obese, should not be ignored.

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Test your knowledge: An older patient who has lived several years in a nursing home and whose clinical status is otherwise stable is suspected on clinical grounds to have protein-energy malnutrition (that is, he has obvious muscle wasting). What is the least useful marker for nutritional status and protein-energy malnutrition?

A. Serum albumin B. Serum prealbumin C. Serial weights with progressive weight loss D. Serum cholesterol E. Total lymphocyte count

Answer to "Test your knowledge:" The answer is E. No nutritional marker is both highly sensitive and specific; all are affected by nonnutritional factors. Total lymphocyte count, although incorporated into some mathematically derived nutritional indices and very easy to obtain from the complete blood count, has not proven to be a useful nutritional marker and is

affected by immune status changes.

The best marker for the early detection of protein-energy malnutrition in frail older adults probably is progressive, involuntary weight loss. Weight measurements are notoriously inaccurate in nursing homes, and weight fluctuations can be caused by changing fluid status; but if a progressive decline is noted (5% in one month, 7% in three months, or 10% in six months), the patient must be evaluated for its cause.

A serum albumin concentration below 3 mg/dL (30 g/L) is a powerful marker for mortality risk, but not necessarily for protein-energy malnutrition. The serum albumin level is also affected by: hydration status (a repeat albumin level obtained after rehydration in an ill patient may be more representative of the patient's protein stores); body position (recumbency lowers serum albumin by about 0.5 mg/dL); hepatic disease; renal disease causing protein loss; malabsorptive syndromes; and inflammatory cytokine production (turning off the hepatic synthesis of albumin in favor of other acute reactant proteins). Endogenous albumin has a half-life of about 20 days and therefore responds slowly to changes to intake alone.

Total serum cholesterol below 160 mg/dL in a frail older adult is likely to be a result of poor nutritional intake and has been associated with increased morbidity. Low prealbumin levels can be caused by protein-energy malnutrition. Its short half-life of two to three days may make prealbumin a more sensitive marker of improving renutrition and anabolism (although the clinical examination is probably equally good but often neglected). Using prealbumin in the general assessment of nursing home residents has not yet proven useful. Falsely elevated levels are common in renal dysfunction.

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Engineering gene expression and protein synthesis by modulation of nuclear shape

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The current understanding of the relationships between cell shape, intracellular forces and signaling, nuclear shape and organization, and gene expression is in its infancy. Here we introduce a method for investigating gene-specific responses in individual cells with controlled nuclear shape and projected area. The shape of the nuclei of primary osteogenic cells were controlled on microfabricated substrata with regiospecific chemistry by confining attachment and spreading of isolated cells on adhesive islands. Gene expression and protein synthesis were altered by changing nuclear shape. Collagen I synthesis correlated directly with cell shape and nuclear shape index (NSI), where intermediate values of nuclear distension (6 < NSI < 8) promoted maximum synthesis. Osteocalcin mRNA, a bone-specific differentiation marker, was observed intracellularly by using reverse transcription *in situ* PCR at 4 days in cells constrained by the pattern and not detected in unconstrained cells of similar projected area, but different NSI. Our data supports the concept of gene expression and protein synthesis based on optimal distortion of the nucleus, possibly altering transcription factor affinity for DNA, transport to the nucleus, or nuclear matrix organization. The combination of microfabricated surfaces, reverse transcription *in situ* PCR, and NSI measurement is an excellent system to study how transcription factors, the nuclear matrix, and the cytoskeleton interact to control gene expression and may be useful for studying a wide variety of other cell shape/gene expression relationships.

patterning | cell size | osteocalcin | reverse transcription–PCR | photolithography

Cell morphology has a profound effect on a range of cellular events, such as proliferation (1–3), differentiation (4–7), cytoskeletal organization (8), and presumably gene expression. Changes to the cytoskeleton lead to altered stress levels imparted on the nucleus (9–11) and could affect organelle and DNA organization and distribution, ultimately altering cell function. For example, the rate of albumin secretion from hepatocytes can be altered by constraining cell size on patterned culture surfaces (3). In human epidermal kareatinocytes cell shape can modulate between terminal differentiation and proliferation (4). Furthermore, cells can be forced to enter the apoptotic cascade when the area on which the cell is allowed to spread is constrained (1). One proposed mechanism for the transduction of cell shape information into gene expression is through mechanical forces transmitted by means of the direct link of the cytoskeleton to the nucleus (9, 12), and in particular to nuclear matrix proteins (NMPs) such as NMP-1 and NMP-2 (13). These architectural transcription factors, which are components of the nuclear scaffold, induce changes in DNA supercoiling and can interact directly with gene promoter sequences (14). This interaction between the nuclear architecture and regulation of transcription or DNA topography raises the question of whether gross deformation of the cell, and hence its nucleus, can modulate the NMP/DNA interactions and gene expression. However, the details in the cascade of mechanical events involving cell morphology, cytoskeletal organization, intracellular signaling, nuclear shape, nuclear matrix organization, promoter geometry, and gene expression are poorly understood.

We report a method for both precisely controlling cell and nuclear shape and measuring *in situ* mRNA expression in an effort to help elaborate these mechanisms. Microfabrication and fluidic techniques have been used to control cell adhesion and spreading on the μm scale and have proven useful for asking basic questions in cell biology (1, 3, 8, 15–19). Although previous studies have used surfaces with spatially resolved chemistry to study cell shape/function relationships and their effect on protein synthesis (1, 3), studies examining *in situ* gene expression (mRNA) and protein synthesis as a function of the cell projected area and nuclear shape have not been published. Therefore, the methods developed in this work were designed to assess cell and nuclear shape-dependent changes in gene expression and protein synthesis of individual cells. An interpenetrating polymer network of poly(acrylamide) and polyethylene glycol [p(AAm-*co*-EG)] grafted to silane-derivatized glass was used as a nonadhesive surface chemistry amenable to the thermal cycling of PCR (20). Combining photolithographic and photopolymerization techniques, we created islands of adhesive surface chemistry [an amine-terminated silane that preferentially adsorbs vitronectin (18)] surrounded by the p(AAm-*co*-EG) interpenetrating polymer network. A range of geometric shapes and sizes were created to impart different mechanical environments within different cells maintained on the same culture surface. Because of our interest in bone, we patterned primary bone-derived cells from rat calvaria and probed for type I collagen (ColII) synthesis and osteocalcin (OC) mRNA expression to determine whether constraining the projected area and nuclear shape of a bone-derived cell altered the time required for differentiation into the osteoblast phenotype. We chose ColII and OC because they are markers of osteoblast differentiation, and NMPs (e.g., Cbfa1, a transcriptional activator of osteoblast differentiation) have been shown to bind to the promoter region of genes for both OC and ColII (13).

Materials and Methods

Surface Preparation. Surfaces were prepared as described in detail by Thomas *et al.* (8) to generate a pattern of *N*-(2-aminoethyl)-3-aminopropyl-trimethoxysilane (EDS; United Chemical Technologies, Bristol, PA) islands with a p(AAm-*co*-EG) background (20). The spatial distribution of the chemistry on these substrata was confirmed by using time-of-flight secondary ion MS with imaging (8). Cell attachment and spreading could be confined to the adhesive islands of the slide, which ranged in area from 75

Abbreviations: NMP, nuclear matrix protein; p(AAm-co-EG), poly(acrylamide-co-ethylene glycol); ColII, type I collagen; OC, osteocalcin; EDS, *N*-(2-aminoethyl)-3-aminopropyl-trimethoxysilane; NSI, nuclear space index; RT, reverse transcription.

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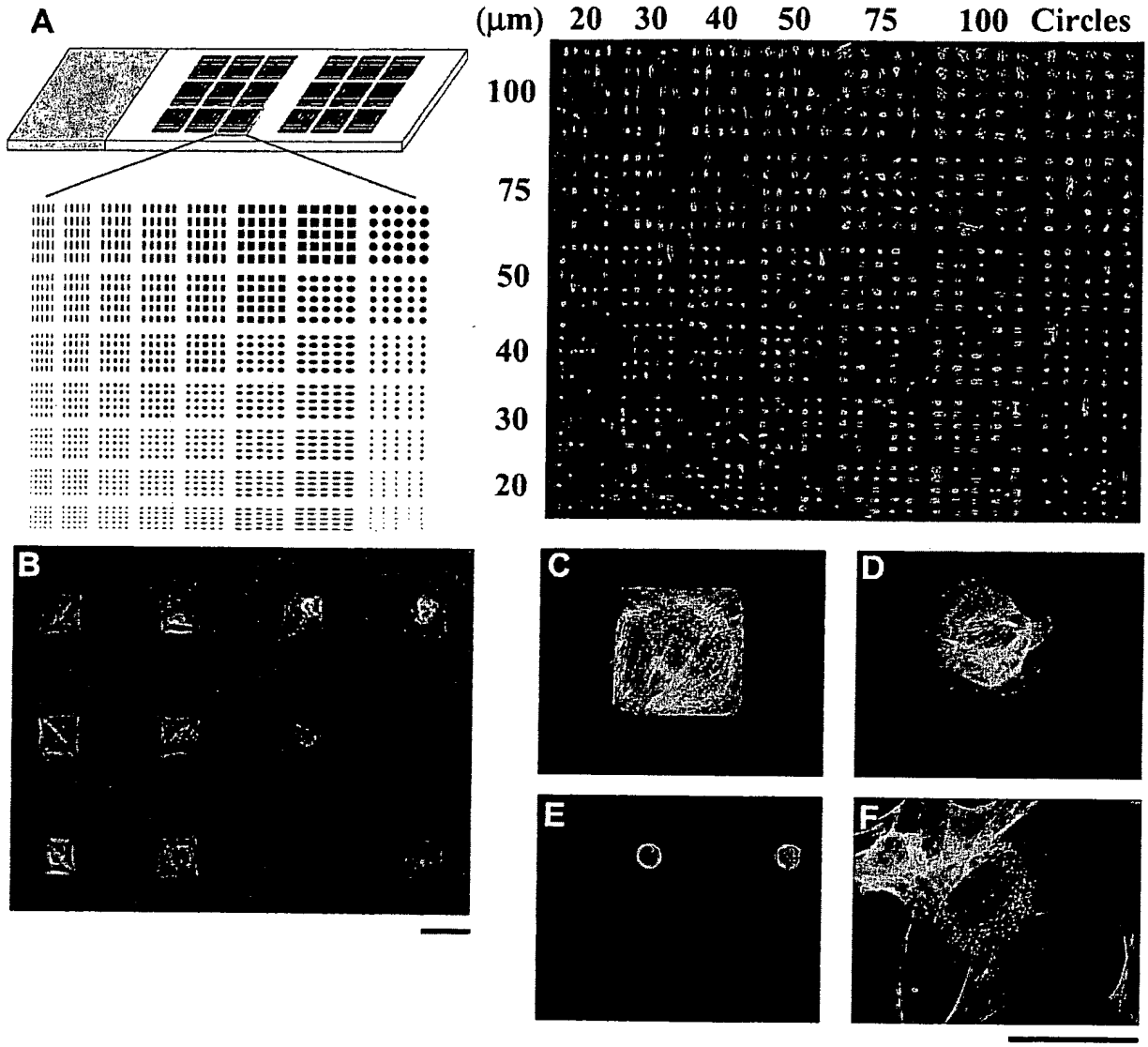

Fig. 1. (*A Left*) Multidomain patterning on a 1 × 3-inch glass slide. The array shown is repeated 18 times on the slide. Two groups of nine arrays were patterned so that control RT *in situ* PCR and hybridization groups could be run on the same slide as experimental groups. (*A Right*) A montage of phase-contrast images of bone-derived cells patterned on one multidomain array on a 1 × 3-inch glass slide. [Reproduced with permission from ref. 8 (Copyright 1999, American Society of Mechanical Engineers).] (*B*) Phase-contrast image of individual cells on 80-μm square islands. (*C–F*) Cells stained for F-actin with phalloidin-Oregon green. Cells on large patterns, 100-μm square (*C*) and 80-μm circle (*D*), arranged their cytoskeletons to conform to the patterned features, whereas cells seeded on 20-μm circles (*E*) showed diffuse staining indicative of a disorganized cytoskeleton. (*F*) Cells seeded on unpatterned EDS surfaces showed organized cytoskeletons, but the orientation was different than those on the constraining islands. (Scale bars = 100 μm.)

to 10,000 μm^2 (8). Alternatively, some experiments were performed on patterned slides prepared in an identical manner with adhesive square EDS islands with areas of 400, 1,600, 3,600, 64,000, or 10,000 μm^2. Homogeneous EDS samples were used as control surfaces for the subsequent experiments.

Cell Culture and Molecular Probes. Primary rat bone cells were isolated from the calvaria of 10- to 12-day-old rats (Harlan–Sprague–Dawley) following methods described by Whitson *et al.* (21). Patterned slides were sterilized with 70% ethanol, dried, and rinsed with PBS. A 2-well chamber system (Falcon 4102) was attached to the slide to confine cells to two separate regions of the pattern. Rat bone cells were plated at a density of 5.0×10^4 cells/cm^2 and maintained in culture before fixation. Patterned cultures were fixed/permeabilized (ORTHO Permea Fix, Ortho Diagnostics) (22), dehydrated with a graded series of ethanol, and rinsed with chloroform to ensure complete dehydration. F-actin staining was performed by blocking samples with BSA and incubating them for 1 h with 5 units/ml Oregon Green 488 phalloidin (Molecular Probes) in PBS. Immunostaining for CollI was performed to examine expression kinetics (2, 4, and 8 days) by using 1:3,000 (vol/vol) monoclonal anti-CollI (C-2456; Sigma). In some experiments, cells were labeled with BrdUrd followed by immunostaining to determine the percentage of cells proliferating on the patterned substrata. BrdUrd labeling was performed for 24 h after 1, 2, and 5 days in culture. Samples were viewed by using phase-contrast and epifluorescence microscopy (Nikon

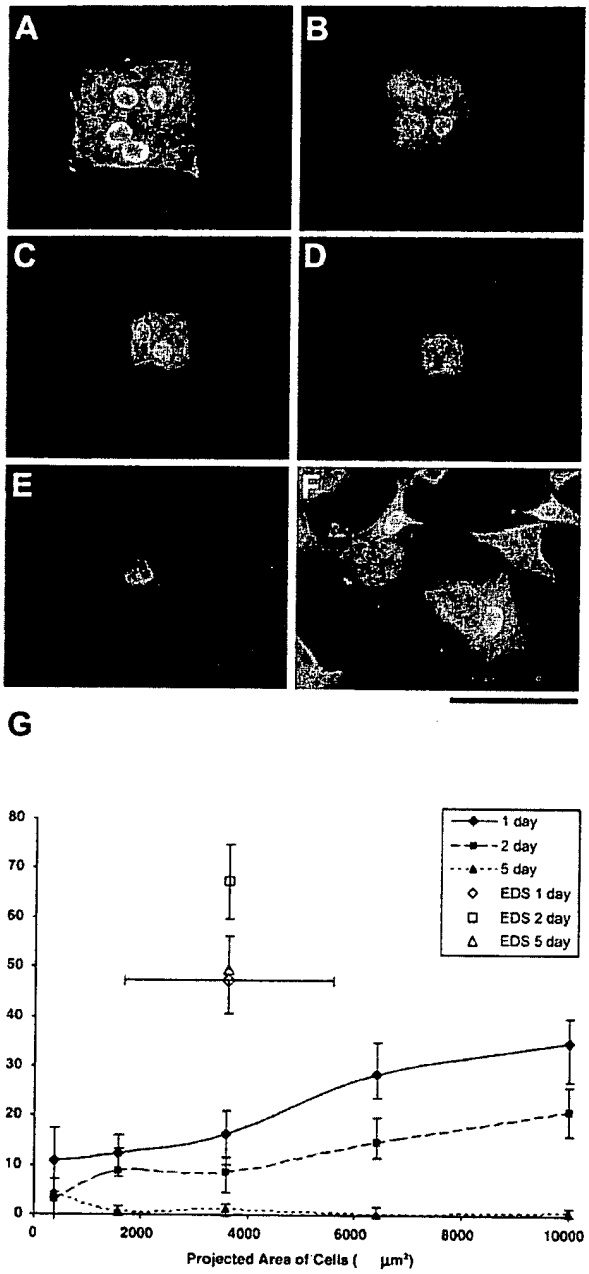

Fig. 2. Images of cells cultured for 5 days on substrates with square islands with side lengths of (*A*) 100 μm, (*B*) 80 μm, (*C*) 60 μm, (*D*) 40 μm, and (*E*) 20 μm, and (*F*) homogeneous EDS surfaces (unconstrained). Cells were labeled with BrdUrd (green nuclei) to indicate DNA synthesis and counterstained with ethidium homodimer (red) for determination of the number of cells in any given island. Counterstained images were enhanced to illustrate the shape of cells. (Scale bar = 100 μm.) (*G*) The percentage of islands with only one cell that was actively proliferating versus projected area. Cells were exposed to BrdUrd for 24 h, labeling all cells that proliferated during that exposure period.

Eclipse TE300), and images were captured by using Oncor IMAGE software and a Sensys high-resolution, cooled charge-coupled device camera (Photometrics, Tucson, AZ).

Nuclear Morphology Measurement. Bone cells from rat calvaria were seeded onto EDS/interpenetrating polymer network-patterned slides and EDS-modified slides. At 24 h in culture, cells were fixed with anhydrous MeOH at −20°C for 10 min, treated with RNase A (10 μg/ml in PBS, Sigma) for 1 h, and stained with ethidium homodimer-1 (4 μM in PBS for 15 min, Molecular Probes). Slides were rinsed with PBS between steps. Two to four drops of SlowFade (Molecular Probes) were placed on the slides, which were then affixed with 150-μm-thick glass spacers (to prevent cell compression) and a 1-oz coverslip. Nuclear morphology measurements were collected with a Zeiss LSM-510 confocal microscope equipped with a 543-nm HeNe laser (80-μm pinhole diameter, optical slice <1.5 μm, ×40 oil objective, numerical aperture 1.0). Gain settings were kept constant throughout all measurements to ensure measurement repeatability. Nuclear height was measured by recording the focus position at the top and bottom of the stained nuclei, and an image was captured at the focus position with maximal nuclear area in the *x-y* plane. Using IPLAB (Scanalytics, Billerica, MA), this image was then segmented and the projected nuclear area was calculated by using a photomask for scale calibration. The nuclear space index (NSI) was calculated as maximal nuclear area in the *x-y* plane divided by nuclear height.

Synthesis of Riboprobes. Digoxigenin-labeled cRNA probes to rat OC were prepared by using the DIG RNA Labeling Kit (Roche Molecular Biochemicals). The 257-bp OC fragment was subcloned into pTAdv by using PCR with the primers 5′-TGC AAA GCC CAG CGA CTC TGA G-3′ and 5′-TTG GGC TCC AGG GCA ACA CAT-3′ and a rat tooth cDNA (22). The sequence was then inserted into the pSPT18 plasmid and sequenced to determine the insert orientation. The cDNA plasmid was transcribed with T7 or SP6 RNA polymerase to create the 257-bp antisense or sense riboprobes, respectively. Digoxigenin-labeled cRNA probes were synthesized in the presence of digoxigenin-dUTP by "run off" transcription, resulting in labeled cRNA probes for *in situ* PCR. This reaction product was elecrophoresed on an agarose gel to verify the probe length and blotted onto a nylon filter to verify digoxigenin incorporation and determine probe concentration.

***In Situ* Reverse Transcription (RT) and PCR.** To detect the low level of expressed OC mRNA (below the 0.161 pg/10,000 μm^2 detection limit) in single cells isolated on patterned substrata, low copy mRNA was amplified by using RT followed by *in situ* PCR. Hybridization was performed by using the digoxigenin-labeled cRNA probes prepared from the 257-bp OC fragment. Briefly, experimental samples were treated with DNase overnight at 37°C in a humid chamber and reverse-transcribed by using the Gene Amp *In Situ* PCR System 1000 (Perkin–Elmer) with random hexamers. Each patterned array of cells (Fig. 1*A*) was covered with an AmpliCover disk and clip (Perkin–Elmer), allowing for experimental and control reactions to be performed on the same slide. The cycle had the following format: denature at 95°C for 1 min, anneal at 56°C for 2 min, and extend at 72°C for 1.5 min (30 cycles). See Fig. 4*A* for an outline of the experimental procedure and the controls used for RT *in situ* PCR. The positive control group received no DNase treatment and resulted in the amplification of the DNA sequence of interest, and the negative control did not receive RT or PCR. After RT-PCR, hybridization was performed at 42°C overnight in the GeneAmp 1000 by using 300 ng/ml of the cRNA probe in hybridization solution. Samples were washed and blocked before probe detection, which was performed by using anti-digoxigenin mAb conjugated with rhodamine (Roche Molecular Biochemicals) diluted 1:1,000 in blocking solution. Samples were viewed by using phase-contrast and epifluorescence microscopy (Nikon Eclipse TE300), and images were captured by using Oncor IMAGE software and a Sensys high-resolution, cooled

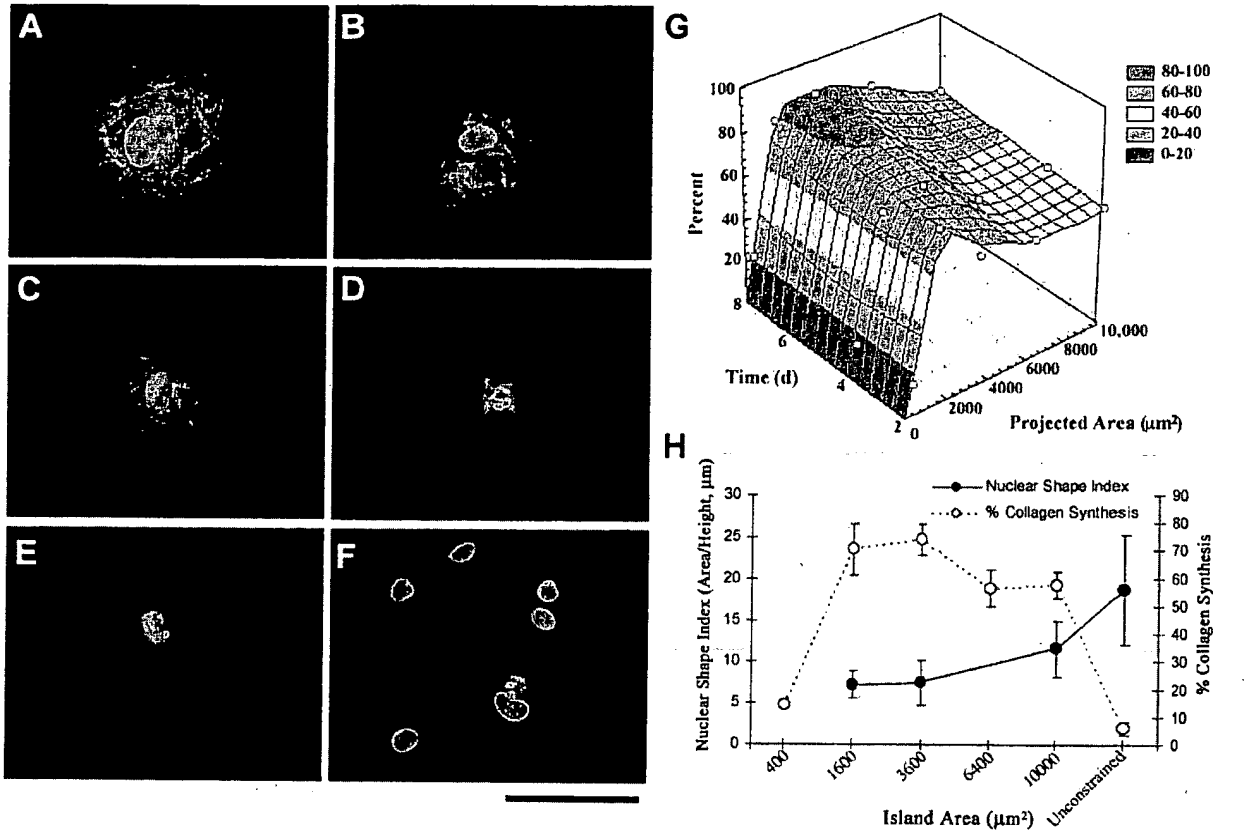

Fig. 3. After 4 days in culture, immunostaining for CollI (green) was performed on substrates with square islands with side lengths of (*A*) 100 μm, (*B*) 80 μm, (*C*) 60 μm, (*D*) 40 μm, and (*E*) 20 μm, and (*F*) homogeneous EDS surfaces (unconstrained). Ethidium homodimer was used as a nuclear counterstain (red). (Scale bar = 100 μm.) (*G*) A three-dimensional plot of the percentages of single cells producing CollI are shown versus projected area and time. Cell exposure to patterned adhesive islands between 1,600 and 3,600 μm^2 resulted in the highest CollI expression levels. (*H*) A dual ordinate plot of NSI (area/height), a metric of nuclear spreading, and CollI synthesis indicate an inverse relationship.

charge-coupled device camera (Photometrics). All images were taken at the same magnification and intensity scaling.

Results

Cell Patterning. When cells were plated on the patterned surfaces, they attached and spread on the adhesive (EDS) islands, assumed the shape of the underlying pattern (Fig. 1 *A* and *B*), and oriented their cytoskeleton to the features on which they were cultured (Fig. 1 *C–F*). Cells were nonadherent to the interpenetrating polymer network.

Cell Proliferation, CollI Synthesis, and Nuclear Shape. Fluorescent images of cells proliferating on islands of various dimensions taken after day 5 of exposure are shown in Fig. 2 *A–F*. Proliferation was greatly restricted for cells confined to smaller islands compared with unpatterned EDS (Fig. 2 *A–G*). Independent of the specific cell projected area, proliferation rates decreased significantly at 1-, 2-, and 5-day time points when compared with unpatterned EDS surfaces that did not limit cell spreading ($P < 0.5$, multiple ANOVA, Newman–Keuls post hoc test). After 5 days, proliferation essentially stopped, and the smallest island sizes (400, 1,600 and 3,600 μm^2) demonstrated the lowest proliferation rates at each time point (Fig. 2*G*) ($P < 0.5$). CollI synthesis was also affected by the island size. CollI synthesis was strongly coupled to cell projected area, with a significantly higher percentage of cells synthesizing CollI when the island projected area was ≥1,600 μm^2 compared with 400 μm^2 EDS islands or control homogeneous EDS surfaces (Fig. 3 *A–G*). The temporal variation in CollI synthesis as a function of cell projected area is given in Fig. 3*G* as a three-dimensional plot. Maximum CollI synthesis occurred between 1,600 and 3,600 μm^2 at each time point and increases with time. Examination of the proliferation (BrdUrd) and the CollI production results provided a kinetic profile of the transition from cell growth and division to phenotypic expression of extracellular matrix production. Islands that reduced the percentage of proliferating cells also decreased the activation time for CollI synthesis (Figs. 2*G* and 3*G*). However, most significantly the level of CollI synthesis correlated to a metric of nuclear shape (area/height) (Fig. 3*H*), where intermediate values of NSI (6–8) and projected cell area (1,600 and 3,600 μm^2) demonstrated maximum CollI synthesis. Embedded in these data are the fact that unconstrained cells on EDS had mean projected areas (3,626 ± 1,948 μm^2; mean ± SD) in the range for maximum CollI synthesis for cells on patterned substrata (1,600–3,600 μm^2); however, these unconstrained cells had very low values of CollI synthesis and a high number of cells (≈50%) proliferating (Figs. 2*G* and 3*G*). The major difference between these unconstrained and constrained cells of similar area was that the NSI was significantly different (Fig. 3*H*).

RT *in Situ* PCR and Hybridization. *In situ* hybridization without RT-PCR resulted in no OC signal detection at 4 days in culture; therefore, RT-PCR was necessary for signal amplification and was performed as described in Fig. 4*A*. Cells cultured on

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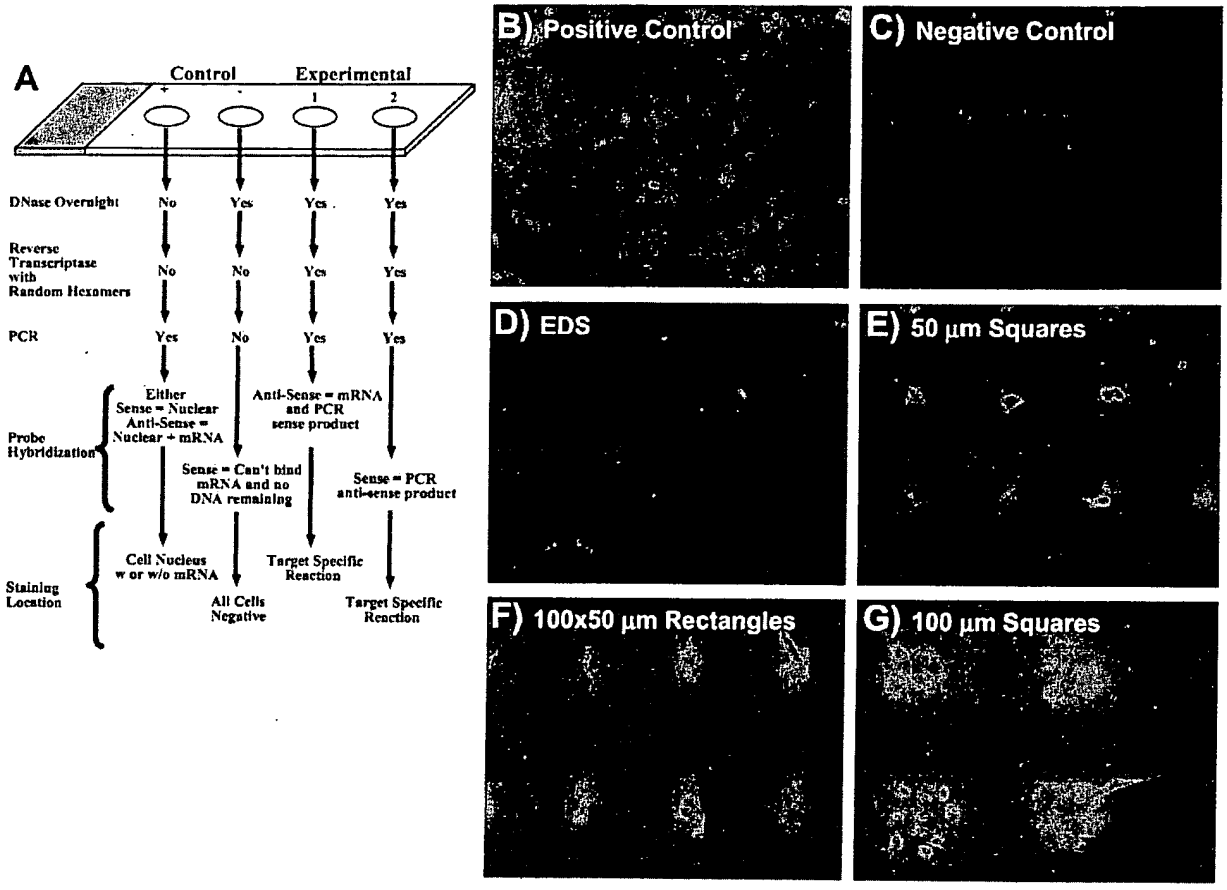

Fig. 4. (*A*) Schematic of the steps necessary for the RT *in situ* PCRs with positive and negative controls. (*B–G*) *In situ* hybridization for OC after RT-PCR for 4-day cultures. (*D*) Control homogeneous EDS. (*B*) Positive RT-PCR/hybridization control, cells on homogeneous EDS. (*C*) Negative RT-PCR/hybridization control, cells on homogeneous EDS. (*E–G*) cells on patterned surfaces: (*E*) 50-μm square; (*F*) 100 × 50-μm rectangle; and (*G*) 100-μm square. (Scale bar = 100 μm.)

homogeneous EDS samples did not stain positively for OC mRNA (Fig. 4*D*). However, cells maintained on the EDS/p(AAM-*co*-EG)-patterned surface stained strongly for OC mRNA (Fig. 4 *E–G*), particularly in their nuclei, indicating active OC mRNA synthesis. The positive control (Fig. 4*B*) shows random spatially distributed nuclear staining; the result of amplification of the OC sequence found in the native DNA and the negative control resulted in low background staining (Fig. 4*C*). The drawback of performing PCR amplification of OC mRNA was the loss of quantitative information, resulting in only the determination of cells either producing or not producing OC mRNA. However, comparison of cells with similar projected areas and different NSI, >15 for unconstrained cells on EDS (Fig. 4*D*) and ≈6–8 for cells patterned on 50-μm square islands (Fig. 4*E*), the mRNA for OC is clearly detected only in the latter.

Discussion

Insight into the impact of cell shape on cell function is key for the understanding of the regulation of gene expression in all cell types, including those in the disease state (12). For example, loss of cell growth dependence on cell shape and surface contact in anchorage-dependent cells is a hallmark of tumor cells *in vivo* and has been induced *in vitro* (23). However, methods for the examination of how the mechanical and chemical factors of the cytoskeleton and the nuclear matrix integrate into a higher-order system are just beginning to emerge. Therefore, the creation and improvement of tools that aid in this research are critical to the success of these efforts.

The micropatterned surfaces used in these studies significantly affected the proliferative and protein synthetic activities of bone-derived cells. A bone-derived cell population was cultured on EDS/p(AAm-*co*-EG)-patterned surfaces with island areas ranging from 75 to 10,000 μm^2. Differentiation into the osteoblast phenotype was determined by a decrease in cell proliferation, CollI synthesis, and the initiation of OC mRNA expression detected by using *in situ* hybridization procedures. Cytoskeletal staining of cells cultured on these patterned surfaces revealed that cells on islands <400 μm^2 did not form stress fibers, whereas cells on larger islands organized their F-actin filaments preferentially at the periphery of the cell. The island domains also had a marked effect on cell proliferation, essentially abolishing cell division on islands of all sizes by day 5. This diminished proliferative capacity was concurrently accompanied by a marked increase in bone-specific protein expression (e.g., CollI) on the patterned surfaces. However, the NSI was the critical metric in distinguishing the amount of protein synthesis of cells spread to the same degree.

Constraining cell spreading and nuclear shape can effectively decrease the time required for phenotypic expression and prompt osteoblasts to begin expressing OC after only 4 days in culture. OC typically is expressed in cultures isolated from rat calvaria after the mineralization process has begun, with mRNA

expression detectable after ≈10 days in culture and protein secretion detectable after ≈15 days in culture (24). Regardless of the adhesive region geometry and cell projected area, OC mRNA was observed in cells cultured on all patterned surfaces that limited cell spreading. The staining was observed in the cytoplasm and localized to the nuclear regions of the cells, which is indicative of active transcription of the OC gene. Cells cultured on control homogeneous EDS surfaces did not exhibit staining above negative control background levels, indicating that OC mRNA was not being expressed in cells cultured on unpatterned surfaces after 4 days. For cells with similar projected area, but different NSI, only cells constrained on the patterned substrata with NSI between 6 and 12 stained positive for OC mRNA.

Collectively, the decreased cell proliferation, the synthesis CollI and mRNA for OC, and nuclear shape data signify a shift from cell growth to differentiation. Patterned cells are forced to cease dividing and differentiate at a significantly earlier time point than unpatterned cells. One hypothesis to explain the mechanism of this effect involves the continuous physical linkage between cytoskeletal elements and the nuclear matrix, by means of extracellular matrix-integrin engagement, which can alter DNA topography and transcription (14). The osteoblast phenotype has been influenced by morphology, external forces, cytoskeletal organization, the extracellular matrix, and nuclear matrix architecture (14, 25). Additionally, several nuclear matrix-specific proteins such as NMP-2 (also identified as Cbfa1 or Osf2) have been shown to bind to the promoter region of the OC gene (13). Therefore, changes in the nuclear matrix architecture could affect the degree of promoter supercoiling and bending and result in altered transcriptional activity of OC. Similarly, NMP-1 (or YY1), which is not osteoblast-specific but binds the OC promoter, is also found in the nuclear matrix fractions of osteoblasts and is recognized as a complex DNA binding/bending protein with multiple roles (13). Both NMP-1 and NMP-2 have been characterized as DNA binding and DNA bending proteins whose involvement in OC expression has been identified but not fully elaborated. However, the continuous link between the extracellular matrix, cytoskeleton, nuclear matrix, and promoter region of the OC gene indicates the importance of cell/nuclear morphology and intracellular signaling in OC gene activation (13, 26). Potentially, engagement of α_2 integrins with CollI and subsequent activation of the mitogen-activated protein kinase pathway (MAPK)/MAPK kinase leads to NMP-2 activation and expression of the OC gene (26). Because NMP-2 binds to the OCN promoter region, optimal NSI may represent the appropriate spatial organization of the promoter amenable to the activated NMP-2. This linkage represents a possible mechanism by which OC expression could be prematurely initiated through alteration of the nuclear morphology as defined by the NSI metric.

These phenomena linking cell shape with nuclear architecture are not osteoblast-specific. Investigations of NMPs and how they are linked to the cytoskeleton have already led to a better understanding of nuclear pores, matrix-associated regions of DNA, and how molecular components of the nucleus are integrated into the machinery of replication, transcription, and posttranscriptional events (7, 27).

These culture surfaces have a number of conceivable applications in the fields of biotechnology and medicine. First, because they allow cell shape and area to be controlled as an independent variable, they are invaluable as experimental platforms for the study of cell shape/gene expression hierarchies. Second, because these surfaces enable the fundamental control of cell differentiation and proliferation, we hope to map the micropatterning technique to the surface modification of biomaterials to control cell behavior at the interface. For example, a more rapid differentiation of bone cells contacting the biomaterial may lead to stronger and quicker integration of the implant. One also can exploit these microfabricated surfaces to create cell-based sensors or cell culture systems that are spatially addressed on a temporal basis for evaluation of lead drug formulations on cells with a more reproducibly defined phenotype.

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CMF608-a novel mechanical strain-induced bone-specific protein expressed in early osteochondroprogenitor cells.

Segev O, Samach A, Faerman A, Kalinski H, Beiman M, Gelfand A, Turam H, Boguslavsky S, Moshayov A, Gottlieb H, Kazanov E, Nevo Z, Robinson D, Skaliter R, Einat P, Binderman I, Feinstein E.

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Microarray gene expression analysis was utilized to identify genes upregulated in primary rat calvaria cultures in response to mechanical force. One of the identified genes designated CMF608 appeared to be novel. The corresponding full-length cDNA was cloned and characterized in more details. It encodes a putative 2597 amino acid protein containing N-terminal signal peptide, six leucine-rich repeats (LRRs), and 12 immunoglobulin-like repeats, 10 of which are clustered within the C-terminus. Expression of CMF608 is bone-specific and the main type of CMF608-positive cells is mesenchymal osteochondroprogenitors with fibroblast-like morphology. These cells reside in the perichondral fibrous ring of La Croix, periosteum, endosteum of normal bone as well as in the activated periosteum and early fibrous callus generated postfracture. Expression of CMF608 is notably absent from the regions of endochondral ossification. Mature bone cell types do not produce CMF608 with the exception of chondrocytes of the tangential layer of the articular cartilage, which are thought to be under constant mechanical loading. Ectopic expression of CMF608 in HEK293T cells shows that the protein is subjected to post-translational processing and its N-terminal approximately 90 kDa polypeptide can be found in the conditioned medium. Ectopic expression of either the full-length cDNA of CMF608 or of its N-terminal region in CMF608-negative ROS17/2.8 rat osteosarcoma cells results in transfected clones displaying increased proliferation rate and

the characteristics of less-differentiated osteoblasts compared to the control cells. Our data indicate that CMF608 is a unique marker of early osteochondroprogenitor cells. We propose that it could be functionally involved in maintenance of the osteochondroprogenitor cells pool and its down-regulation precedes terminal differentiation.

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